

## Plant Regeneration from Cell Suspension Culture of Rice Varieties Khao Dawk Mali 105 and Suphanburi 1

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### ABSTRACT

Cell suspension cultures of rice varieties Khao Dawk Mali 105 and Suphanburi 1 were tested in eight media. Both varieties had successfully established in N6 medium supplemented with 2.0 or 4.0 mg/l 2,4-D. Rice variety Suphanburi 1 showed higher cell growth rate than variety Khao Dawk Mali 105 while the percentage of cell viability and embryogenic cell were not significantly different. However, the regeneration of plantlets from cell suspension derived-calli of rice variety Khao Dawk Mali 105 was significantly higher (83.3%) than that of variety Suphanburi 1 (67.8%).

**Key words:** Cell suspension, Rice, Khao Dawk Mali 105, Suphanburi 1

### INTRODUCTION

The viability of cell suspension cultures is an important system for cell growth investigations, secondary metabolite production (Hilliou *et al.*, 1999), as well as for mass propagation of plants. For protoplast technology, leaf mesophyll is an ideal source for protoplast isolation in dicotyledonous species. However, the leaf is not practicable tissue for protoplast isolation in monocotyledonous species especially in rice. The rice leaf consists a numerous of silica cells and cork cells whose walls can not be digested enzymatically. Consequently, there are no reports on protoplast isolation and culture for rice leaf explants. Establishment of cell suspensions consisting of single cells and small clusters of rapidly-dividing cells thus seem as a critical step in the isolation of rice protoplasts (Datta *et al.*, 1990; Utomo *et al.*, 1995). Embryogenic cell suspension cultures are also a source and target for gene transformation (Nandadeva *et al.*, 1999),

consequently, transgenic plants have been obtained by microprojectile-mediated transient of cell suspension in several varieties of rice (Zhang *et al.*, 1996; Sathishkumar *et al.*, 1997; Biswas *et al.*, 1998).

It has been found that several factors affect growth, maintenance and regeneration of rice cell suspension. In this study the basal medium, plant growth regulators, carbohydrate sources and inorganic nitrogen were investigated for establishment of the Thai rice varieties Khao Dawk Mali 105 and Suphanburi 1.

### MATERIALS AND METHODS

#### Plant materials

Two commercial Thai rice varieties, namely Khao Dawk Mali 105 (KDML 105) and Suphanburi 1 were selected for the establishment of cell suspensions. The seeds were obtained from the Phatumthani Rice Research Station, Phatumthani Province.

### Medium compositions

Nine promising media were selected and used for cell suspension initiation of the two rice varieties. The medium compositions were presented in Table 1.

### Callus initiation

Mature seeds were dehusked, sterilized and cultured on MS medium supplemented with 2.0 mg/l 2,4-D, 100 mg/l casein hydrolysate and 30 g/l sucrose for callus formation. The cultures were incubated at  $25\pm 2^{\circ}\text{C}$ , 16h photoperiod for 3 weeks.

### Initiation and establishment of cell suspension cultures

Cell suspension cultures of both varieties were initiated from callus (Figure 3A). Approximately 1.0 g f.wt. were transferred into 100 ml Erlenmeyer flasks, each flask containing 20 ml liquid medium (Table 1). The cultures were incubated on a rotary platform shaker (120 rpm),

at  $25\pm 2^{\circ}\text{C}$ , 16h photoperiod. All suspensions were replaced with 10 ml of fresh medium every 4 days. After 4 weeks of culture the suspension cultures were subcultured every 7-day intervals by transferring the equivalent of 2 ml packed cell volume (pcv) of cells together with 20 ml of spent medium into a 250 ml Erlenmeyer flask containing 20 ml of fresh medium.

### Assessment of cell growth

One ml pcv of cells from suspension cultures were subcultured into 50 ml ND2 medium, dispensed in 250 ml side-armed Erlenmeyer flask using sterilized plastic pipette. The cultures were incubated on rotary platform shaker (120 rpm) at  $25\pm 2^{\circ}\text{C}$ , 16 h photoperiod. Pack cell volumes were recorded every 4-day intervals for 24 days.

### Determination of cell viability

Suspension cell viability was evaluated using FDA staining. Cell viability was determined

**Table 1** Composition of medium used for rice cell suspension cultures.

Basal Medium	AA2	R2	ND2	ND4	NP1	NP3	MSD2	MSP1	LS2.5
LS									+
MS							+	+	
N6			+	+	+	+			
AA	+								
R2		+							
2,4-D (mg/l)	2.0	2.0	2.0	4.0	-	-	2.0	-	2.5
Kinetin	0.2	-	-	-	-	-	-	-	-
GA <sub>3</sub>	0.1	-	-	-	-	-	-	-	-
Picloram	-	-	-	-	1.0	3.0	-	1.0	-
Proline (g/l)	-	-	1.38	1.38	1.38	1.38	-	1.38	-
MES (g/l)	-	-	1.0	1.0	1.0	1.0	-	-	-
Sorbitol (g/l)	-	-	1.5	1.5	1.5	1.5	-	1.5	-
Sucrose	30	20	10	10	10	10	30	-	30

LS: Linsmaier and Skoog's medium, LS2.5: modified LS, MS: Murashige and Skoog's medium, MSD2 and MSP1: modified MS; N6: Chu's medium; ND2, ND4, NP1 and NP3: modified N6; AA: Muller and Grafe's medium; R2: Ohira's medium. AA2 medium was sterilized through membrane (0.2  $\mu\text{m}$  pore size). The other media were sterilized by autoclaving at  $120^{\circ}\text{C}$  for 20 min. All media were adjusted pH at 5.8 before sterilization.

using an ultraviolet fluorescence inverted microscope (Nikon "Diaphot TMD"). Cell sample was transferred onto a microslide, covered with a cover slip. Viable cells labeled with FDA were visible under UV light at 495 nm and exhibited green-yellow fluorescence.

#### Determination of embryogenic cells

The suspension consisted of two cell types, embryogenic and non-embryogenic. Six-week-old suspension cells were transferred onto a microslide, covered with a cover slip. Both types of cell were identified and recorded under inverted microscope.

#### Plant regeneration from callus derived-suspension culture

Eight-week-old microcalli (2-4 mm diam.) were selected and transferred to 100 ml Erlenmeyer flask. Approximately 100 calli were put in each flask. The calli were rinsed twice with 200 ml sterile water to remove the old suspension medium. Thirty calli were plated in each 9 cm plastic petri dish containing 20 ml regeneration medium (MS medium supplemented with 2.0 mg/l BAP; 1.0

mg/l NAA and 30g/l sucrose). The plates were incubated at  $25\pm 2^{\circ}\text{C}$ , 16 h photoperiod.

## RESULTS AND DISCUSSIONS

#### Influence of culture medium on establishment of cell suspensions

Calli cultured in liquid AA2, R2, MSD2 or LS2.5 media did not give suspensions (Table 2). Both varieties did not respond in these media. They failed to proliferate and turned brown within a few days. Although AA2 medium has been used successfully to maintain rice suspensions both for japonica and indica groups (Khehra, 1995; Tang, 1995; Lee, 1996; Azhakanandam, 1999). Similarly, R2 medium has been employed for suspension cultures in indica varieties (Torriso and Zapata, 1992; Basu *et al.*, 1997). Cultures in NP1, NP3 or MSP1 media gave single cells, but further cell division and development was not observed. Both varieties showed some responsiveness on the modified N6 medium, ND2 and ND4 media gave elongated-cells and some embryogenic clusters. These clusters developed to calli. It has been noted that N6 medium supplemented with 2 and 4 mg/l

**Table 2** Response of rice variety KDML 105 and Suphanburi 1 in suspension culture medium.

Medium	Variety KDML 105	Variety Suphanburi 1
AA2	-	-
R2	-	-
ND2	++	++
ND4	++	++
NP1	+	+
NP3	+	+
MSD2-	-	-
MSP1	+	+
LS2.5	-	-

- : Calli gave no suspensions.

+ : Calli gave suspensions consisting predominantly of elongated cells which did not grow after subculture; cells died within 2 weeks.

++: Calli gave suspensions consisting of a mixture of elongated cells and embryonic clusters

2,4-D (ND2, ND4) gave better suspensions than the medium supplemented with 1 and 3 mg/l picloram (NP1, NP3; Table 2). Consequently, ND2 medium was chosen for suspension initiation of KDML 105 and Suphanburi 1 varieties.

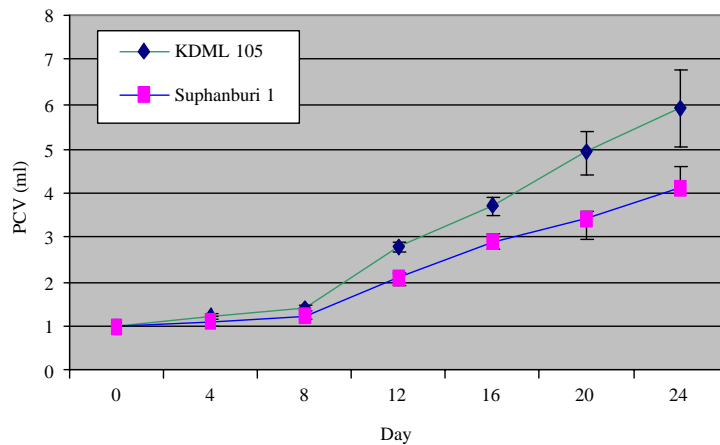
### Assessment of cell growth

Single cells of suspension cultures divided and formed small clumps of cells after 2 weeks of culture (Figure 3B-C). Microcalli with 2-3 mm diameter. were observed by 3 weeks of culture

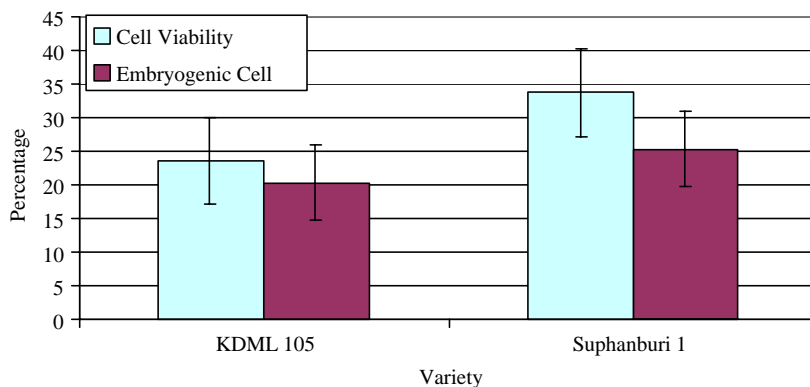
(Figure 3D-E). Rice variety KDML 105 gave a higher growth rate than variety Suphanburi 1 in ND2 medium (Figure 1).

### Determination of cell viability

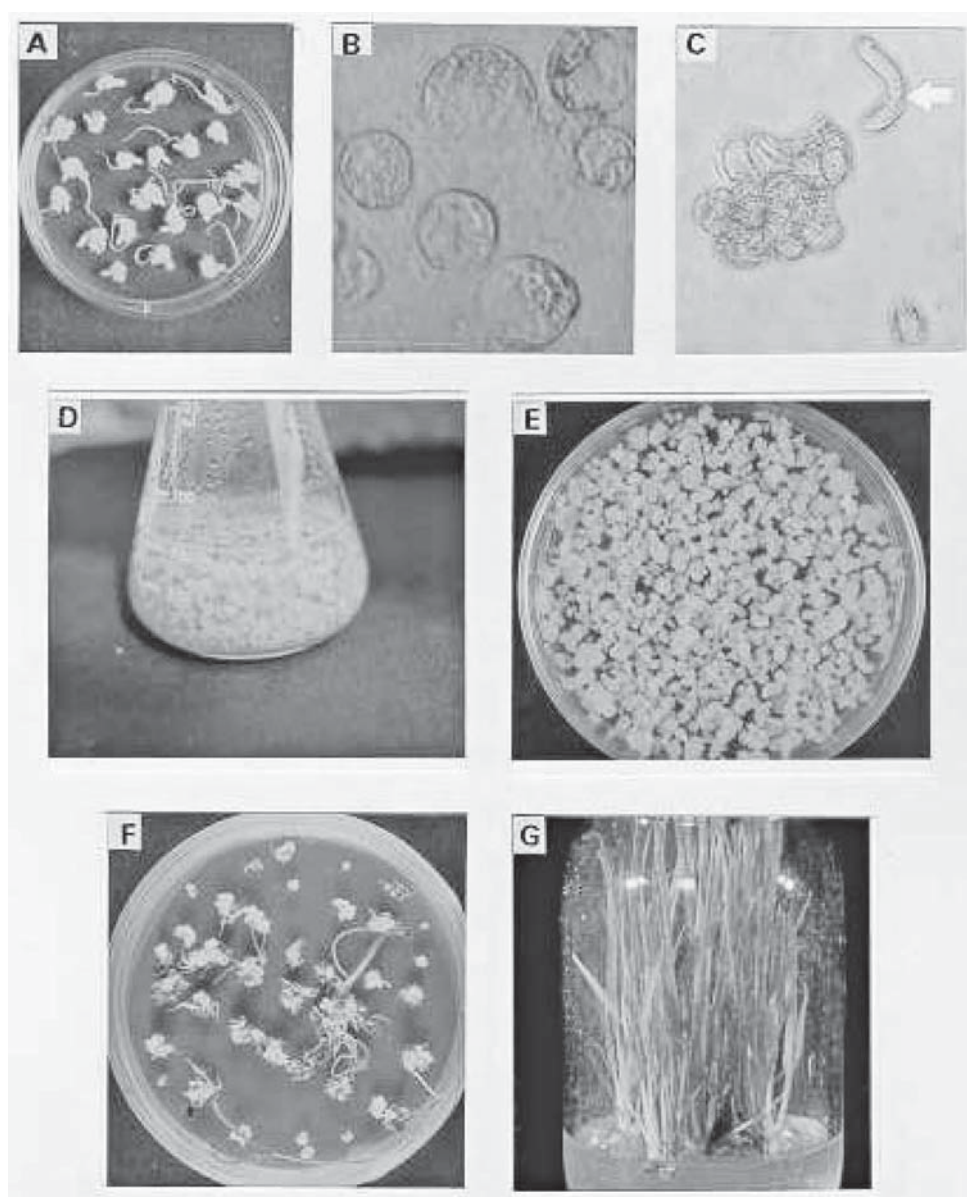
Cells of rice variety Suphanburi 1 had a higher percentage of viability than variety KDML 105 after 6 weeks of culture in ND2 medium (Figure 2). It was noted that the viable cells consisted of both round-and-elongate-shaped cells.



**Figure 1** Growth rate of cell suspensions cultured in ND2 medium of rice varieties KDML 105 and Suphanburi 1. Data represent means of packed cell volume (pcv), bars represent standard deviations (SD) based on 3 replicates, 4 samples were used in each replicate.



**Figure 2** Percentage of cell viability and embryogenic cells. Data represent means of percentage, bars represent standard deviations (SD) based on 3 replicates, 10 samples were used in each replicate.



**Figure 3** Regeneration of rice plants varieties Khao Dawk Mali 105 and Suphanburi 1 through cell suspension culture system. A: Calli derived from mature seeds, B: Single cells isolated from calli, C: Cluster of embryogenic cells and non-embryogenic cell (arrow), D: Calli developed from single cells in liquid medium, E: Calli used for plant regeneration, F: Plantlets regenerated from calli, G: Establishment of plantlets on regeneration medium.

**Table 3** Percentage of plant regeneration from callus derived-suspension.

Variety	(%) Regeneration
Khao Dawk Mali 105	83.33 $\pm$ 9.63 <sup>a</sup>
Supunburi 1	68.71 $\pm$ 8.44

Data represent means of percentage of regeneration  $\pm$  standard deviations (SD) based on 4 replicates and 180 calli were used in each replicate. a: Significant at  $P < 0.05$

### Determination of embryogenic cells

It has been noted that the suspension consisted of two types of cells, embryogenic and non-embryogenic cells. Embryogenic cell has a round-shaped, large condense nucleus and small volume of vacuoles while non-embryogenic cell has a long-shaped, small nucleus and contains a large volume of vacuoles (Figure 3C). It was found that only the embryogenic cell has a potency to divide and forms a cell cluster. The rice variety Suphanburi 1 showed slightly higher percentage of embryogenic and viable cells than variety KDML 105 (Figure 2).

### Plant regeneration from callus derived-suspension cultures

Media containing 2,4-D completely inhibited plant regeneration in rice. Thus the calli in suspension cultured in ND2 medium (supplemented with 2 mg l<sup>-1</sup> 2,4-D) were thoroughly rinsed prior to transfer to regeneration medium. Rice variety KDML 105 showed a higher regeneration ability than variety Suphanburi 1 (Table 3). Plantlets also formed roots on regeneration medium without further subculture (Figure 3F-G), and they were well established when transferred to the soil.

### CONCLUSIONS

1. Cell suspension culture of rice varieties Khao Dawk Mali 105 and Suphanburi 1 could be established in N6 medium supplemented with 2.0 mg/l 2,4-D.

2. Cell proliferation rate of Suphanburi 1 variety was higher than variety Khao Dawk Mali 105.

3. Percentage of plant regeneration from callus-derived cell suspension was high (83.3% and 68.7% for rice variety Khao Dawk Mali 105 and Suphanburi 1, respectively).

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